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Acute phase serum amyloid A induces proinflammatory cytokines and mineralization via toll-like receptor 4 in mesenchymal stem cells☆

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ABSTRACT

The role of serum amyloid A (SAA) proteins, which are ligands for toll-like receptors, was analyzed in human bone marrow-derived mesenchymal stem cells (hMSCs) and their osteogenic offspring with a focus on senescence, differentiation and mineralization. In vitro aged hMSC developed a senescence-associated secretory phenotype (SASP), resulting in enhanced SAA1/2, TLR2/4 and proinflammatory cytokine (IL6, IL8, IL1β, CXCL1, CXCL2) expression before entering replicative senescence. Recombinant human SAA1 (rhSAA1) induced SASP-related genes and proteins in MSC, which could be abolished by cotreatment with the TLR4-inhibitor CLI-095. The same pattern of SASP-resembling genes was stimulated upon induction of osteogenic differentiation, which is accompanied by autocrine SAA1/2 expression. In this context additional rhSAA1 enhanced the SASP-like phenotype, accelerated the proinflammatory phase of osteogenic differentiation and enhanced mineralization. Autocrine/paracrine and rhSAA1 via TLR4 stimulate a proinflammatory phenotype that is both part of the early phase of osteogenic differentiation and the development of senescence. This signaling cascade is tightly involved in bone formation and mineralization, but may also propagate pathological extraosseous calcification conditions such as calcifying inflammation and atherosclerosis.

1. Introduction

The primary response to inflammation, infection and tissue injury is mediated by the innate immune system via toll-like receptors (TLR1–6). TLRs bind a variety of ligands to transduce signals via receptor-associated kinase systems such as p38 activation, NFκB nuclear translocation and STAT signaling cascades, which trigger the release of pro-inflammatory cytokines like IL1β, IL6 and members of the CCL and CXCL families of cytokines (Kang and Lee, 2011; Brown et al., 2011) that recruit cells of the adaptive immune system if necessary.

The TLR family of receptors is tightly involved in the modulation of functions of mesenchymal and other stem cells (Rolls et al., 2007). Bone marrow-derived mesenchymal stem cells (MSCs) are the principal source of bone regeneration. MSCs have been reported to express TLR1–6 and MSC treatment with a cocktail of pro-inflammatory cytokines especially upregulates TLR2, 3 and 4 expression in mice and humans (Delarosa et al., 2012). The role of TLR in MSC biology has not yet been completely unraveled and variably contrasting effects of TLR activation on MSC biology have been reported. TLR2 activation using the ligand Pam3Cys followed by NFκB nuclear translocation completely abolished MSC multipotent differentiation capacity in mice (Pevsner-Fischer et al., 2007), while depending on the time frame and type of activation osteogenic differentiation was either inhibited or enhanced (Huang et al., 2014; Mo et al., 2008; Raicevic et al., 2012; Chang et al., 2013; Zhao et al., 2011). Hence the complete picture of the role of TLR expression in MSC biology is just emerging (Delarosa et al., 2012).

Abbreviations: CXCL1, chemokine (C–X–C motif) ligand 1; frz, frizzled; hMSCs, human bone marrow-derived mesenchymal stem cells; IBSP, integrin-binding sialoprotein; IL1B, interleukin 1 beta; IL6, interleukin 6; IL8, interleukin 8; OP, osteopontin; OPG: TNFRSF11B, tumor necrosis factor receptor superfamily, member 11b; p16: CDKN2A, cyclin-dependent kinase inhibitor 2A; ROR2, receptor tyrosine kinase-like orphan receptor 2; RUNX2, runt-related transcription factor 2; SAA1, serum amyloid A1; SAA2, serum amyloid A2; SASP, senescence-associated proinflammatory phenotype; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; WNT5A, wingless-type MMTV integration site family, member 5A.

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Bone regeneration and bone healing are accompanied by an initial inflammatory reaction following injury and an initial burst of growth factors released by platelets from fracture hematoma (Kolar et al., 2010; Gerstenfeld et al., 2003). Pro-inflammatory stimuli have been reported to enhance osteogenic differentiation and bone healing but may also inhibit bone formation depending on the duration and the specific signaling stimulus (Mo et al., 2008; Raicevic et al., 2012; Chang et al., 2013; Zhao et al., 2011). Moreover, osteogenic differentiation itself stimulates the expression of TLR2 and 4 in hMSC (Kovacevic et al., 2008). In murine and human MSCs TNFα stimulation and NFκB activation have shown very inconsistent results with respect to osteogenic differentiation and bone healing depending e.g. on the origin of MSC (adipose tissue versus bone marrow) and also the recruitment and migration phases compared to later phases of bone healing (Pevsner-Fischer et al., 2007; Huang et al., 2014; Raicevic et al., 2012). Mineralization is an endpoint of osteogenic differentiation and bone formation. However extra-osseous mineralization plays an important role in chronic inflammatory and aging-associated degenerative diseases such as atherosclerosis and sclerosing bone metastases (Hofbauer et al., 2014). We have recently described the expression of WNT5A in TNF and LPS-treated skeletal precursors and a subtle analysis of WNT5A effects on murine MSC described an amplification of pro-inflammatory signals downstream WNT5A (Rauner et al., 2012). Immunohistochemistry analyses of atherosclerotic lesions also revealed marked expression of WNT5A in such areas, consistent with the hypothesis that atherosclerosis is a chronic inflammatory disease (Christman et al., 2008). Given the fact however that treatment with anti-TNF antibodies under certain circumstances could stop arthritis but not the extraosseous bone formation, there is still a different trigger to be found for the inflammatory self-sustaining loop and this pathological form of mineralization.

Replicative senescence is another condition that can be preceded and propagated by a very similar cascade of proinflammatory signals, which is called a senescence-associated proinflammatory phenotype (SASP) (Velarde et al., 2013). Since this SASP can "infect" neighboring cells a condition can be spread that is capable of impairing fundamental processes of regeneration and lineage commitment.

Serum amyloid A proteins 1 and 2 (SAA1 and 2) are the major acute-phase proteins systemically secreted by the liver in response to inflammation, trauma or infection but local expression was also detected in various other tissues such as the bone, cartilage and vessels, as well as in inflamed tissues and serum in osteoarthritis and atherosclerosis (Kovacevic et al., 2008; Vallon et al., 2001; de Seny et al., 2013; Meek et al., 1994; Eklund et al., 2012). SAA proteins are encoded by the *SAA1* and *SAA2* genes, which seem to be coordinately expressed (Thorn et al., 2004). Their transcription is induced by glucocorticoids and cytokines like TNFα, IL1α, IL1β and IL6 (Kovacevic et al., 2008; Vallon et al., 2001; Thorn et al., 2004; Hagihara et al., 2004). In turn autocrine SAA was described to amplify the proinflammatory environment by again stimulating cytokine expression, e.g. IL6, IL8, and CXCL1, and to induce the expression of metalloproteinases at the tissue level (Vallon et al., 2001; de Seny et al., 2013). The signal transduction pathways SAA proteins can address are transmitted via TLR2 and 4 receptors and via the formyl peptide receptor FPRL1 in various cell systems (Cheng et al., 2008; Sandri et al., 2008; Dong et al., 2011; Su et al., 1999).

We show here that SAA is locally expressed in hMSC, its expression is enhanced during both in vitro aging and normal osteogenic differentiation and that by binding to TLR4 it further contributes to a self-sustaining pro-inflammatory amplification loop that consists of TLR4 activation via p38 kinase, NFκB activation by p65 phosphorylation and downstream stimulation of pro-inflammatory cytokines. This micromilieu, when transient, can either support wnt/frz-related osteogenic differentiation (Baron and Kneissel, 2013) or induce cellular aging and senescence because it resembles a SASP phenotype. Hence SAA via TLR4 activation may both be an enhancer of osteogenic differentiation and a trigger of inflammation-associated calcification.

2. Materials and methods

2.1. Cell culture and in vitro aging

Human MSCs were obtained from the bone marrow of femoral heads according to the described protocol (Ebert et al., 2009) after total hip arthroplasty due to osteoarthritis and/or hip dysplasia. The procedure was approved by the local Ethics Committee of the University of Würzburg. Briefly, bone marrow preparations were washed with Dulbecco's modified Eagle's medium (DMEM/F12) (Life Technologies GmbH, Darmstadt, Germany) supplemented with 10% heat-inactivated FCS (Biochrom GmbH, Berlin, Germany), 1 U/ml penicillin, 100 μg/ml streptomycin (all Life Technologies GmbH), and 50 μg/ml ascorbate (Sigma-Aldrich GmbH, Munich, Germany) and centrifuged at 1200 rpm for 5 min. The pellet was reconstituted in medium and washed 4 times, and the supernatants of the washing steps containing the released cells were collected. Cells were centrifuged and cultivated at a density of 3×10^8 cells per 150 cm^2 culture flask. Adherent cells were washed after 2 days and cultivated until confluence. Cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air. For long term cultivation, cells were expanded at 70–90% confluence by trypsinization with 1× trypsin–EDTA (Life Technologies GmbH) and reseeding in a split ratio of 1:3 relative to the cell culture flask surface. This procedure was repeated until cells stopped proliferation and hMSC did not become confluent within 3 weeks due to replicative senescence. The time frame of this process was individually different for each of the 8 hMSC donors used. The last, non-confluent passage was defined as "passage x" (Px) (Px MSC1: P8; Px MSC2: P9; Px MSC3: P10; Px MSC4 and 5: P11; Px MSC6: P12; Px MSC7: P15; Px MSC8: P18).

2.2. Osteogenic differentiation of hMSC

Human MSCs were differentiated into the osteoblastic lineage by seeding 1×10^4 cells per cm^2 in 6-well plates for RNA isolation and 4-well Lab-Tek Chamber Slides (Nunc, Wiesbaden, Germany) for histochemical staining. After reaching confluence, medium was replaced by osteogenic medium (OM) consisting of DMEM high glucose, 10% FCS, 1 U/ml penicillin, 100 μg/ml streptomycin (all Life Technologies GmbH), 50 μg/ml L-ascorbic acid 2-phosphate, 1 μM dexamethasone and 10 mM β-glycerophosphate (all Sigma Aldrich GmbH). Control cells were kept in expansion medium. Influence of SAA, SAA in combination with the TLR4 inhibitor CLI-095, and CLI-095 alone on mineralization and alkaline phosphatase (ALP) staining was tested by adding 1 μM rhSAA1 (PeproTech GmbH, Hamburg, Germany) and/or 1 μg/ml of CLI-095 (InvivoGen, Toulouse, France) throughout the osteogenic differentiation process. Medium was changed twice a week.

2.3. Histochemical staining

ALP was stained after 4 days or 2 weeks of differentiation using the Alkaline Phosphatase, Leukocyte Kit 86-C (Sigma Aldrich GmbH) according to the manufacturer's instructions. For the detection of calcium hydrogen phosphate and hydroxylapatite in the extracellular matrix hMSCs were fixed in methanol after 7 days or 3 weeks, stained with alkaline Alizarin Red S (1% w/v) (Chroma-Schmidt GmbH, Stuttgart, Germany) for 2 min and air dried. Microscopic images were taken at room temperature with an Axioskop 2 MOT microscope with a 10×/0.3 Plan Neofluar objective and an AxioCam MRc camera (all Carl Zeiss Microimaging GmbH, Göttingen, Germany). Staining was quantified using the AutMess tool of the AxioVision Software (Carl Zeiss Microimaging GmbH) by analyzing 6 to 20 randomly selected pictures per preparation. Significances were tested with the Wilcoxon signed-ranks test.

2.4. Quantitative real-time PCR

Total RNA was isolated after 24 h of 0.1, 0.5, 1 and 2 μM rhSAA1 treatment, costimulation with 1 μM rhSAA1 and with 1 μg/ml of the TLR4 signaling inhibitor CLI-095 and after 1, 7 and 14 days of osteogenic differentiation with and without 1 μM rhSAA1 treatment by using the NucleoSpin RNA II Purification Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. For cDNA synthesis 1 μg of total RNA was reverse transcribed with Oligo(dT)15 primers and MMLV reverse transcriptase (both Promega GmbH, Mannheim, Germany) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using KAPA SYBR FAST Universal 2× qPCR Master Mix (Peqlab Biotechnologie GmbH) and 0.25 pmol of sequence specific primers obtained from biomers.net GmbH (Ulm, Germany) or Qiagen GmbH (Hilden, Germany) (see Table 1 for primer sequences and PCR conditions). Results were calculated with the efficiency-corrected Ct model (Pfaffl, 2001) with RPS27A as the house-keeping gene. Significance was tested with REST (Pfaffl et al., 2002).

2.5. Western blot

Human MSCs were treated with 1 μM rhSAA1 for 30 and 60 min, respectively and cotreated with the TLR4 inhibitor CLI-095. Cells were washed twice with PBS and harvested in 200 μl lysis buffer (100 μM HEPES, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT) containing cOmplete protease inhibitor (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). 5 μl 10% Nonidet-P40 was added, followed by centrifugation at 13,000 rpm, 4 °C for 30 s. Proteins were quantified by RotiQuant assay (Carl Roth GmbH, Karlsruhe, Germany). 15 μg protein was mixed with 3.75 μl loading buffer (RotiLoad, Carl Roth GmbH) and denaturated by boiling for 5 min. Samples were separated by SDS polyacrylamide gel electrophoresis on 0.8× MDE gels (Biozym, Hess. Oldendorf, Germany), 375 mM Tris, pH 8.8, 0.1% SDS in 192 mM glycine, 25 mM Tris, 0.1% SDS, pH 8.8. Proteins were electroblotted for 2 h at 20 V, 4 °C to a stabilized nitrocellulose membrane (Optitran BA-S, Schleicher and Schuell, Dassel, Germany) using a Mini Protean unit (BioRad, München, Germany). Membranes were blocked with 2.5% nonfat milk powder in TTBS buffer (0.1 M Tris, 150 mM NaCl, 0.1% Tween-20) and incubated with phospho-p38 MAPK (#9215), p38α MAPK (#9217), phospho-NFκB (p65 #3033), and NFκB p65 (#6956) (dilution 1:2000, all Cell Signaling Technology Inc., Leiden, The Netherlands) primary antibodies diluted in blocking solution overnight at 4 °C. Membranes were washed 3× for 15 min with TTBS followed by incubation for 2 h at room temperature with secondary anti-mouse or anti-rabbit IgG-horseradish peroxidase antibodies, respectively (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) diluted 1:2000 in TTBS solution. After another washing 3× for 15 min with TTBS specific staining was detected using the chemiluminescence (ECL) system (VWR International GmbH, Darmstadt, Germany). All bands were densitometrically analyzed with ImageJ.

2.6. Enzyme linked immunosorbent assay

Human MSCs were stimulated with 1 μM rhSAA1 for 1 day or cotreated with 1 μg/ml of the TLR4 inhibitor CLI-095. Supernatants were harvested, diluted 1:20 (controls and CLI-095 treated samples) and 1:50 (rhSAA1 treated samples) and IL-6 and IL-8 were determined with ELISA (IL-6: 900-K16, IL8: 900-K18, both PeproTech GmbH) according to the manufacturer's instructions. 3 independent MSC preparations were used and values were normalized to IL-6 and IL-8 standard curves. Significances were tested with the Mann–Whitney U test.

Table 1
PCR primers and conditions. Primer names, sequences, product lengths, annealing temperatures and GenBank accession numbers are shown.

Gene	Primer	Sequence 5′–3′	Product length (bp)	Annealing temp (°C)	GenBank accession number
CXCL1	CXCL1 qFOR	GAAAGCTTGCCTCAATCCTG	88	57	NM_001511
	CXCL1 qREV	CACCAGTGAGCTTCCTCCTC			
CXCL2	CXCL2 qFOR	AGCTTATTGGTGGCTGTTCC	102	60	NM_002089
	CXCL2 qREV	ACACATTAGGCGCAATCCAG			
IBSP	Hs_IBSP_1_SG	Qiagen sequence		57	NM_004967
IL1B	IL1b qFOR	AAACCTCTTCGAGGCACAAG	169	57	NM_000576
	IL1b qREV	GGCCATCAGCTTCAAAGAAC			
Il6	Il6 qFOR	AAAGCAGCAAAGAGGCACTG	108	60	NM_000600
	Il6 qREV	TTTTCACCAGGCAAGTCTCC			
IL8	IL8 qFOR	CATACTCCAAACCTTTCCAC	165	60	BT007067
	IL8 qREV	TCAAAAACTTCTCCACAACC			
OPG	Hs_TNFRSF11B_1_SG	Qiagen sequence		60	NM_002546
OP	OP qFOR	TATGATGGCCGAGGTGATAG	133	60	NM_001040058
	OP qREV	CATTCAACTCCTCGCTTTCC			
p16	p16 qFOR	TGGACCTGGCTGAGGAGCT	107	58	NM_000077
	p16 qREV	GACCTTCCGCGGCATCTA			
PTGS2	Hs_PTGS2_1_SG	Qiagen sequence		59	NM_000963
ROR2	ROR2 qFOR	CTGTGTGACGTACCCTCGTG	108	59	NM_004560
	ROR2 qREV	AGAAGAAAAGGCAAGCGATG			
RPS27A	RPS27A qFOR	TCGTGGTGGTGCTAAGAAAA	141	60	NM_001135592
	RPS27A qREV	TCTCGACGAAGGCGACTAAT			
RUNX2	Runx2 qFOR	CTTCACAAATCCTCCCCAAG	147	58	NM_001024630
	Runx2 qREV	ATGCGCCCTAAATCACTGAG			
SAA1	SAA1 qFOR	GCAAAGACCCCAATCACTTC	127	57	M23699
	SAA1 qREV	GTACCCTCTCCCCGCTTTG			
SAA2	SAA2 qFOR	CGATCAGGCTGCCAATAAAT	124	60	AB937783
	SAA2 qREV	GCCTCATAGCCAGGTCTCCT			
TLR2	TLR2 qFOR	GAGGCAGCGAGAAAGCG	318	60	XM_005263194
	TLR2 qREV	CCATTGCGGTCACAAGA			
TLR4	TLR4 qFOR	TCCATTTCAGCTCTGCCTTC	114	57	NM_138554
	TLR4 qREV	TGGGACACCACAACAATCAC			
WNT5A	WNT5a qFOR	GAAGCCAATTCTTGGTGGTC	190	60	XM_011534081
	WNT5a qREV	GGCATTCTTTGATGCCTGTC			

3. Results

3.1. SAA expression is associated with in vitro aging of hMSC

To analyze cellular senescence of bone-forming cells in vitro we performed long-term cultivation of hMSC until cells stopped proliferation as previously described (Benisch et al., 2012). The senescent state had been verified by enhanced p16 expression in cells of the last, senescent passage compared to the first passage by Western blot analyses (data not shown). The amount of time and number of passages until growth arrest differed widely between hMSC populations of 8 different donors with 8–15 cumulative population doublings and 8–18 passages, respectively. Assuming that each hMSC population underwent an in vitro aging process in its individual time course, we divided the number of passages until growth arrest into 4 equal quarters (Fig. 1A). RNA was isolated and relative gene expression of SASP-associated genes was analyzed of cells at the end of the 1st, 2nd and 3rd quarters, the last proliferating passage (Px − 1) and the growth-arrested, senescent passage (Px). Gene expression was compared to cells of the first passage (P1) (Fig. 1B). Over the time course of in vitro aging we detected continuously increasing expression of p16 (*CDKN2A*) and SASP genes *IL1B*, *IL6* and *OPG*. An extreme increase in the expression of acute-phase SAA genes *SAA1* and *SAA2* was observed after two-thirds of cultivation time, which continued to a lesser extent until the cells stopped proliferating. Furthermore, we detected increased expression of the SAA receptor *TLR4* in late passages. Other known SASP genes, e.g. *IL8 and CXCL2* (data not shown) were not significantly differentially expressed during in vitro aging.

3.2. rhSAA1 induces a senescence associated secretory phenotype in hMSC through TLR4 signaling

To identify the optimal rhSAA1 concentration hMSCs isolated from 5 individual donors were treated with different doses of recombinant human SAA1 (rhSAA1) for 24 h and *IL-6* expression and *IL-8* expression were quantified by qPCR (Supplementary Fig. 1). 1 μg/ml was chosen as the best concentration and was used in all experiments. To determine if

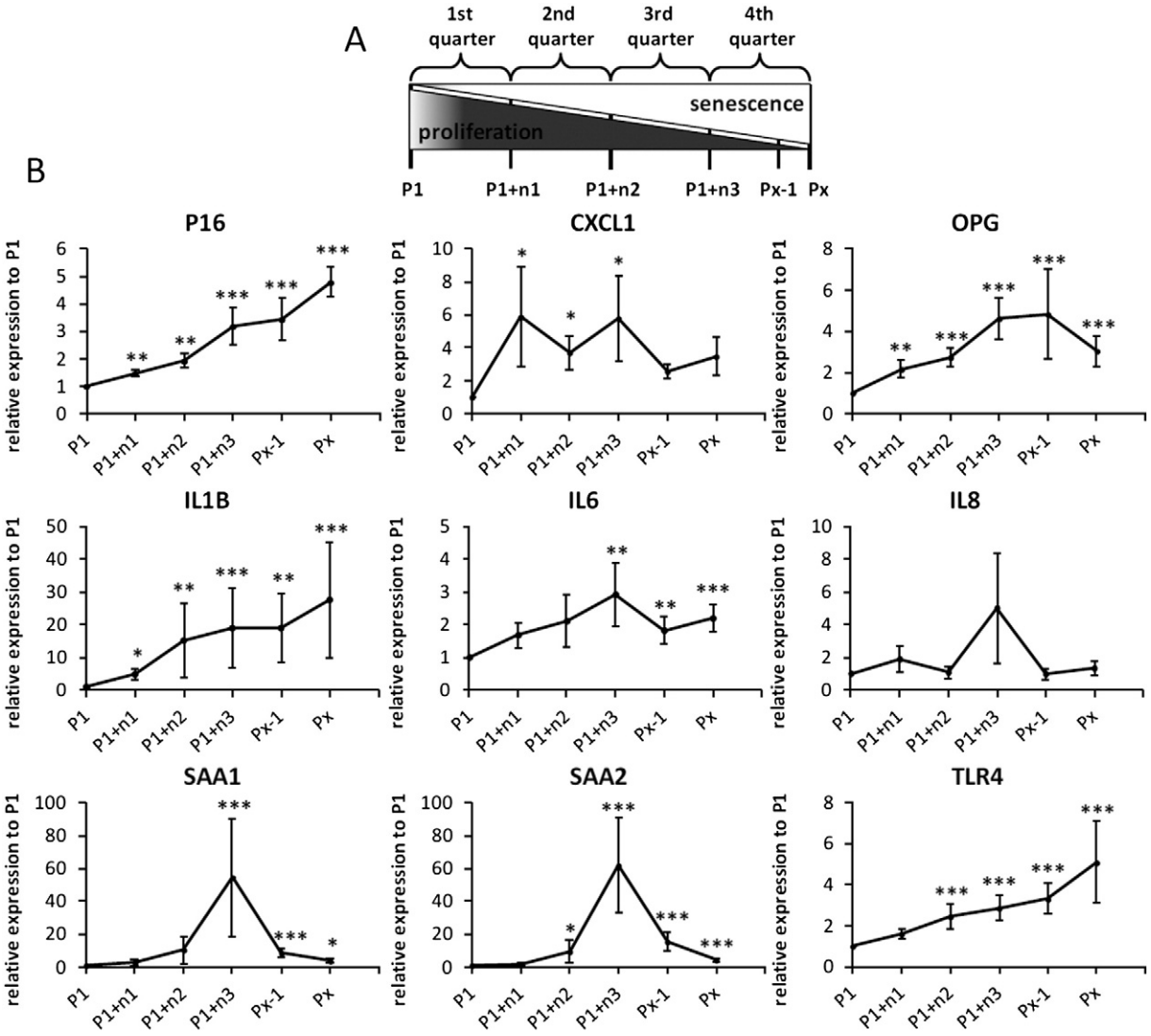

Fig. 1. Long-term cultivation of MSC. Long term cultivated hMSCs develop a senescence-associated secretory phenotype (SASP) over time and show an enhanced expression of acute phase serum amyloids *SAA1* and *SAA2*. (A) HMSCs were cultured and expanded in vitro until they stopped proliferation and entered cellular senescence. The numbers of passages varied until different hMSC populations stopped to proliferate and were divided into 4 quarters. (B) qPCR was performed with mRNA of passages at the end of the 1st quarter (P1 + n1), 2nd quarter (P1 + n2), 3rd quarter (P1 + n3), the last proliferating passage (Px − 1) and the growth-arrested, senescent passage (Px). The first passage (P1) served as control. Dots show the means of ratios compared to P1 of 8 hMSC populations ± SD. Due to undetectable gene expression in P1 of hMSC of one donor *IL8* shows means of 7 hMSC populations. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by REST (Pfaffl et al., 2002). (CXCL1: chemokine (C–X–C motif) ligand 1; IL1B: interleukin 1 beta, IL6: interleukin 6, IL8: interleukin 8; OPG: TNFRSF11B, tumor necrosis factor receptor superfamily, member 11b; p16: CDKN2A, cyclin-dependent kinase inhibitor 2A; SAA1: serum amyloid A1; SAA2: serum amyloid A2; TLR4: toll-like receptor 4).

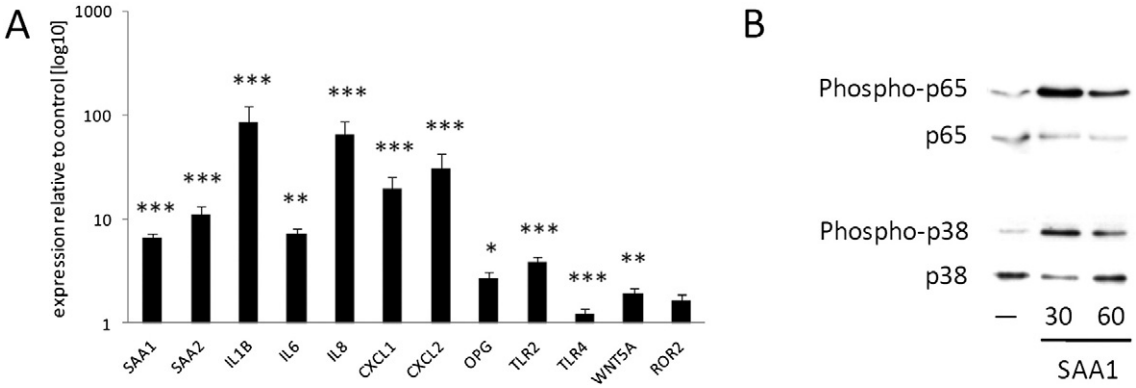

Fig. 2. rhSAA1 signaling pathway and target genes. rhSAA1 induces gene expression of SASP proteins via TLR4 through p38 and NFκB signaling pathways in hMSCs. (A) qPCR of hMSC stimulated with 1 μM rhSAA1 for 24 h. Bars represent the mean change in gene expression ± SD of rhSAA1 treated 6 hMSC populations compared to untreated controls ± SD; *p < 0.05, **p < 0.01, and ***p < 0.001 by REST (Pfaffl et al., 2002). (B) p38 and p65 (NFκB) phosphorylation after treating cells with 1 μM rhSAA1 for 30 and 60 min shown by Western blot. Experiments were repeated twice, and representative stainings are shown. (CXCL1: chemokine (C–X–C motif) ligand 1; CXCL2: chemokine (C–X–C motif) ligand 2; IL1B: interleukin 1 beta, IL6: interleukin 6, IL8: interleukin 8; OPG: TNFRSF11B, tumor necrosis factor receptor superfamily, member 11b; p16: CDKN2A, cyclin-dependent kinase inhibitor 2A; PTGS2: prostaglandin-endoperoxide synthase 2; ROR2: receptor tyrosine kinase-like orphan receptor 2; SAA1: serum amyloid A1; SAA2: serum amyloid A2; TLR2: toll-like receptor 2; TLR4: toll-like receptor 4; WNT5A: wingless-type MMTV integration site family, member 5A).

enhanced expression of *SAA* is only associated with or is an inductor of the development of a senescence associated secretory phenotype (SASP) in hMSC, we treated cells of 6 different donors in P1 with 1 μM rhSAA1 for 24 h and analyzed gene expression by qPCR (Fig. 2A). We could confirm that rhSAA1 induces its own endogenous expression (*SAA1* and *SAA2*) as well as the expression of *IL1B*, *IL6*, *IL8* and *CXCL1*, as described in osteoarthritic fibroblast-like synoviocytes (de Seny et al., 2013). IL-6 and IL-8 secretion could also been quantified after rhSAA1 stimulation by ELISA in 3 different hMSC donors (Fig. 3C). The concentration of IL-6 increased significantly from 7.3 ng/ml to 158 ng/ml after rhSAA1 treatment, while IL-8 was raised significantly from 1.9 ng/ml to 56 ng/ml. In addition we found enhanced expression of the known SASP genes *CXCL2* and *OPG*. We also observed an increase in the expression of SAA receptors *TLR2* and *TLR4* and the non-canonical

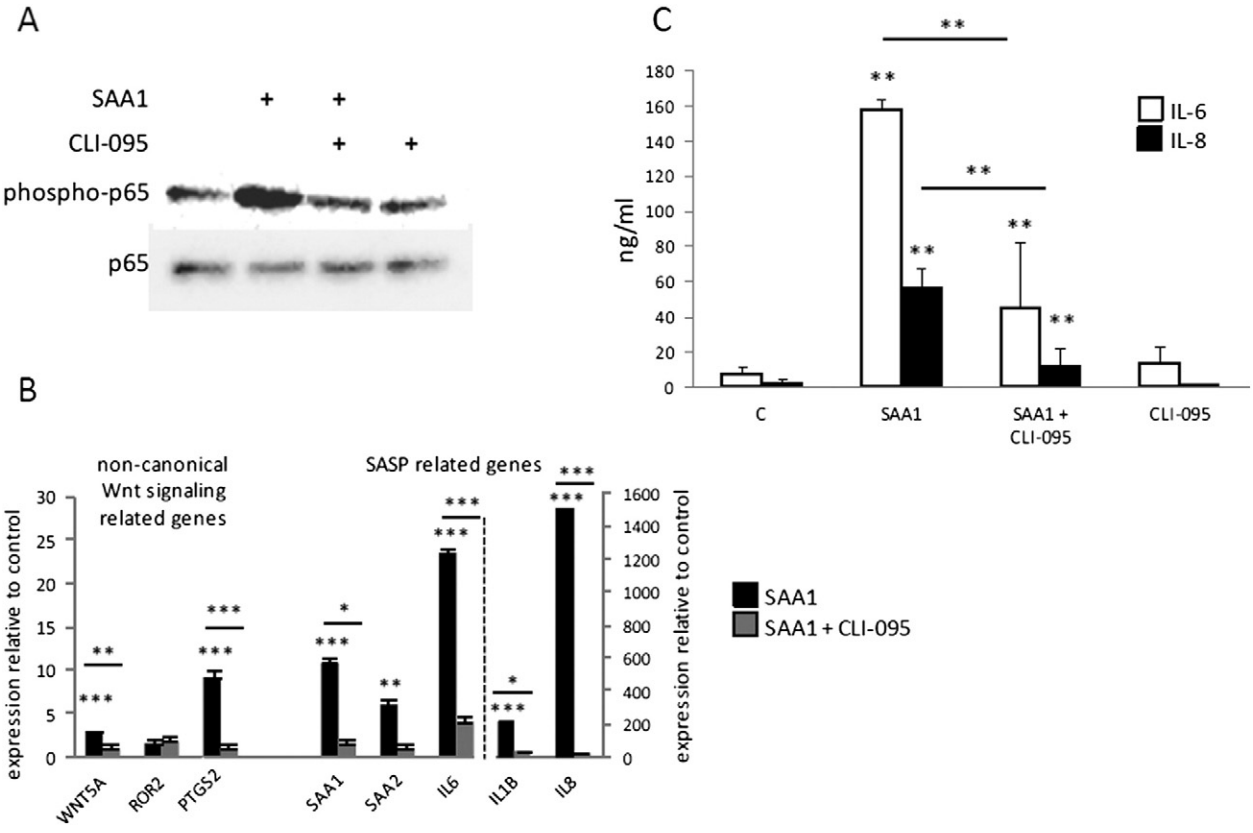

Fig. 3. Toll-like receptor 4 mediates rhSAA1 effects in hMSC. (A) The TLR4 inhibitor CLI-095 diminishes rhSAA1 effects on p65 phosphorylation after 30 min in hMSC. For normalization staining of unphosphorylated p65 is depicted. Experiments were repeated twice, a representative image is shown. (B) Cotreatment of cells with rhSAA1 and the TLR4 inhibitor CLI-095 abrogated rhSAA1 effects on gene expression. Black bars represent the mean change in gene expression ± SD of rhSAA1 treated 4 to 5 hMSC populations compared to untreated controls, gray bars represent the mean change in gene expression + SD of rhSAA1 and CLI-095 cotreated 4 to 5 hMSC populations compared to untreated controls; *p < 0.05, **p < 0.01, and ***p < 0.001 by REST (Pfaffl et al., 2002). (C) Cotreatment of cells with rhSAA1 and the TLR4 inhibitor CLI-095 abrogated rhSAA1 effects on cytokine secretion. IL-6 (white bars) and IL-8 (black bars) were determined in cell culture supernatants of 3 independent hMSC populations treated with rhSAA1 or cotreated with rhSAA1 and the TLR4 inhibitor CLI-095 for 1 day. Values are shown as means + SD (**p < 0.01 by Mann–Whitney U-test).

wnt ligand *WNT5A*. p38 phosphorylation and p65/NFκB phosphorylation were also investigated as mediators of TLR signaling (Brown et al., 2011; DelaRosa and Lombardo, 2010; Yang et al., 2012). We found that p38 and p65 phosphorylation occurred after 30 min treatment with rhSAA1 in 3 different hMSC donors. A representative blot is shown (Fig. 2B) where densitometrical analyses of bands revealed a 9-fold upregulation of p65 phosphorylation and a 24-fold upregulation of p38 phosphorylation after 30 min. Densities of phosphorylated proteins were normalized to unphosphorylated controls.

3.3. rhSAA1 effects are mediated by TLR4 in hMSC

In order to dissect the downstream signaling mechanism of gene regulation of rhSAA1/TLR4 we analyzed if the TLR4 signaling inhibitor CLI-095 was able to abolish rhSAA1 effects on cellular signaling and gene expression. Cotreatment of hMSC isolated from 3 donors with rhSAA1 and the TLR4 inhibitor CLI-095 abolished the phosphorylation of p65/NFκB induced by rhSAA1 alone after 30 min by Western blot. Fig. 3A shows a representative image where rhSAA1 increased p65 phosphorylation more than 3-fold. Cotreatment of cells with rhSAA1 and CLI-095 abrogated this effect (1.2-fold induction). Densities of phosphorylated proteins were normalized to unphosphorylated controls. Co-stimulation of hMSC from 3 to 4 donors with CLI-095 and rhSAA1 significantly abrogated rhSAA1 effects on genes involved in non-canonical wnt-signaling (WNT5A and the AP-1 target gene prostaglandin-endoperoxide synthase 2 (*PTGS2*)) as well as the expression of the SASP genes *SAA1*, *IL6*, *IL1B* and *IL8* (Fig. 3B). On protein levels this effect could be verified by the quantification of IL-6 (Fig. 3C, white bars) and IL-8 (black bars) by ELISA in cell culture supernatants of hMSC from 4 different donors stimulated with rhSAA1 or cotreated with rhSAA1 and the TLR4 inhibitor CLI-095. IL-6 values were significantly abrogated from 158 ng/ml (rhSAA1 treatment) to 45 ng/ml (rhSAA1 plus CLI-095), while IL-8 concentrations were significantly diminished from 56 ng/ml (rhSAA1) to 11 ng/ml (rhSAA1 plus CLI-095). CLI-095 alone had no effect on IL-6 or IL8 protein levels.

3.4. rhSAA1 stimulates non-canonical wnt signaling by inducing WNT5A and accelerates osteogenic differentiation in vitro

To characterize SAA target genes in P1 hMSC we also analyzed the expression of genes relevant for osteogenic differentiation upon stimulation with rhSAA1 and found an increase in *WNT5A* expression and a trend for *ROR2* (Fig. 2A). To analyze if osteogenic effects were more pronounced in conditions promoting osteogenic differentiation we incubated hMSC from 6 different donors with osteogenic medium for 1, 7 and 14 days in the absence and presence of rhSAA1 (Fig. 4). Standard osteogenic media alone significantly stimulated endogenous *SAA1* and *2* expression compared to undifferentiated controls. This effect was enhanced by day 1 but was abolished by day 14 in the presence of exogenous rhSAA1 (Fig. 4). Very similar patterns, e.g. an increase during osteogenic differentiation compared to undifferentiated controls, were observed for *TLR2*, *TLR4*, *ROR2*, *IBSP*, *WNT5a* and *OP* although an increase within the time frame of osteogenic differentiation could only be reported for *OP* and *IBSP* (Fig. 4, black lines). In the presence of rhSAA1 the rise of expression along osteogenic differentiation was shifted to earlier time points, possibly indicating an inflammation-associated acceleration of osteogenic differentiation. Gene expression of osteoblastic markers such as *RUNX2* (runt-related transcription factor 2) and *IBSP* (bone sialoprotein II) was enhanced by rhSAA1 at early time points while *OP* (osteopontin) was not changed (Fig. 4, dotted lines).

3.5. rhSAA1 enhances mineralization during osteogenic differentiation in vitro

In order to clarify if the accelerated and enhanced pro-inflammatory microenvironment leads to enhanced early (4 to 7 days) and late (2 to 3 weeks) functional readouts of osteogenic differentiation we analyzed late in vitro ALP activity and mineralization under continuous rhSAA1 stimulation in 8 different hMSC donors. For early effects and to confirm TLR4 dependent signaling 3 different hMSC donors were stimulated with rhSAA1 and costimulated with the TLR4 inhibitor CLI-095.

After 4 days of stimulation with rhSAA1 in osteogenic differentiation media (OM) ALP activity was significantly enhanced compared to cells cultivated in OM alone (Figs. 5A and B). TLR4 inhibition with CLI-095 significantly abrogated the effect induced by rhSAA1, and CLI-095 alone had no impact on ALP activity (Figs. 5A and B). For long-term effects ALP activity was analyzed after 2 weeks and quantification of the ALP staining revealed no difference between OM and OM + rhSAA1 cultivated cells (data not shown).

After 7 days of stimulating cells with rhSAA1 in OM Alizarin Red S staining visualized the beginning of mineralization, which was completely absent in cells cultivated in OM alone (Fig. 5C). Cotreatment with the TLR4 inhibitor CLI-095 abrogated this effect, which was confirmed by quantitative analysis of the mineralized area (Fig. 5D). CLI-095 alone had no influence on mineralization. For long-term effects, after 3 weeks in OM, we also confirmed a higher mineralization level in rhSAA1 treated cells as visualized by Alizarin Red S staining (Fig. 5E) and quantification of the mineralized area (Fig. 5F).

4. Discussion

Toll like receptors like TLR2 and 4 are important mediators of inflammation, differentiation and aging in bone marrow derived mesenchymal stem cells (hMSCs) (Delarosa et al., 2012). Chronic inflammation has been associated with cellular and organismal aging and the term “inflammaging” has been coined for this phenomenon (Franceschi et al., 2007). Micro-RNAs have recently been discussed to dysregulate TLR activation and the consecutive propagation of a senescence associated secretory phenotype (SASP), which comprises stimulation of pro-inflammatory cytokine secretion such as IL1β, IL8 and of a distinct pattern of metalloproteinases (Brown et al., 2011; Olivieri et al., 2013). Serum amyloid protein is another stimulator of inflammation that is secreted by the liver in large amounts in response to injury and infection. However it can also be locally produced in diseases such as rheumatoid arthritis, osteoarthritis and atherosclerosis (Kovacevic et al., 2008; Vallon et al., 2001; de Seny et al., 2013; Meek et al., 1994; Eklund et al., 2012). TLR2 and TLR4 have been shown to be functional receptors for SAA (Cheng et al., 2008; Sandri et al., 2008). Here we show that SAA is increasingly produced by hMSC during in vitro aging. In this situation SAA expression is associated with the development of a SASP and the rising expression of e.g. IL1β, IL8 and OPG in aging hMSCs (Figs. 1 and 3 and reported in Ren et al. (2013)). In order to establish a causal relationship between those two phenomena we stimulated early passage hMSC with rhSAA1 and we could induce cytokine production after 24 h resembling a SASP phenotype, even in cells that so far have not yet been presenescent. Moreover, rhSAA1 stimulated the endogenous production of SAA1 and 2 thus propagating an autocrine feedback loop, which is capable of sustaining and amplifying a proinflammatory microenvironment. We conclude therefrom that both endogenous and exogenous SAA can stimulate the SASP in natural defense and in disease in hMSC and can propagate cellular aging and replicative senescence. Our results in this model of in vitro aging of hMSC closely resemble the concept established by Rodier and Campisi, which describes the development of an increasingly complex and self-sustaining secretory phenotype that ceases at the very moment of established cellular aging and may have implications for tumor development and other age-associated diseases (Rodier and Campisi, 2011). Endogenous and exogenous SAA may be a novel stimulator and orchestrator of this process, which is a self-sustaining one in several aspects like amplification of the proinflammatory cascade through several players, but also through paracrine spreading of the SASP and of presenescence. One important self-sustaining component is for example inflammasome

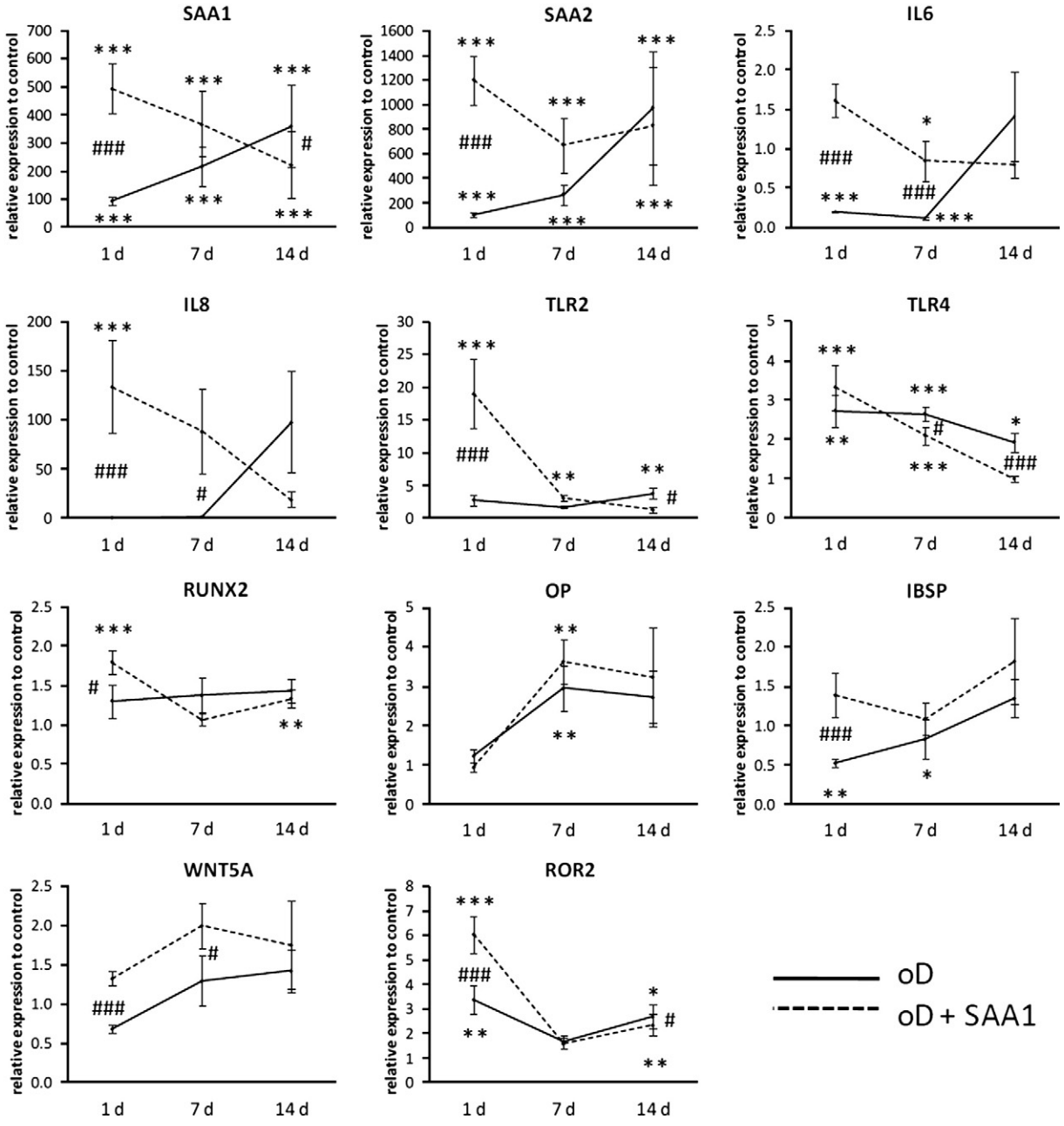

Fig. 4. Osteogenic differentiation and gene expression. rhSAA1 enhances and accelerates mineralization during osteogenic differentiation of hMSC. Gene expression of inflammation-associated genes and osteoblastic markers was analyzed after 1, 7 and 14 days of osteogenic differentiation (black line) and after osteogenic differentiation in the presence of 1 μM rhSAA1 (dotted line). Lines represent the means of changes in expression ± SD of 6 hMSC populations compared to undifferentiated control cells at the respective time point. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Significances were also calculated for the effect of rhSAA1 on osteogenic differentiation ($^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ with REST (Pfaffl et al., 2002)). (IBSP: integrin-binding sialoprotein; IL1B: interleukin 1 beta, IL6: interleukin 6, IL8: interleukin 8; OP: osteopontin; ROR2: receptor tyrosine kinase-like orphan receptor 2; RUNX2: runt-related transcription factor 2; SAA1: serum amyloid A1; SAA2: serum amyloid A2; TLR2: toll-like receptor 2; TLR4: toll-like receptor 4; WNT5A: wingless-type MMTV integration site family, member 5A).

activation, which exerts IL1α and β production, where the former further orchestrates inflammasome activation. Not only is this process self-sustaining but it is also "infectious" as it has been demonstrated by Acosta et al. in a recent paper, where they coined the term "paracrine senescence" (Acosta et al., 2013). We propose that SAA can be added to the players, which can initiate and amplify autocrine and paracrine senescence.

In contrast to chronic TLR activation and inflammation, short time stimulation of the innate immune system may have completely different tasks with respect to tissue maturation and repair. It is well known that bone healing during fracture repair is induced by platelet derived growth factor stimulation after injury, followed by an inflammatory phase that precedes the osteogenic and the remodeling phases (Kolar et al., 2010; Ai-Aql et al., 2008). Osteogenic non-canonical wnt-signaling through WNT5A secretion is stimulated by proinflammatory stimuli (Rauner et al., 2012). We therefore hypothesized that both the endogenous production of SAA and exogenous SAA exposure during injury and infection may modulate the osteogenic differentiation process. During in vitro osteogenic differentiation using established osteogenic media SAA1 and 2 are both stimulated and this is associated with a very similar proinflammatory phenotype observed in our aging model. The peak of the expression is however early in the course of osteogenic differentiation and seems to cease at later time points. When we on top added rhSAA1 to these routine osteogenic differentiation experiments we observed a marked acceleration of the induction of osteogenic differentiation and the pro-inflammatory phenotype (Fig. 4). We also

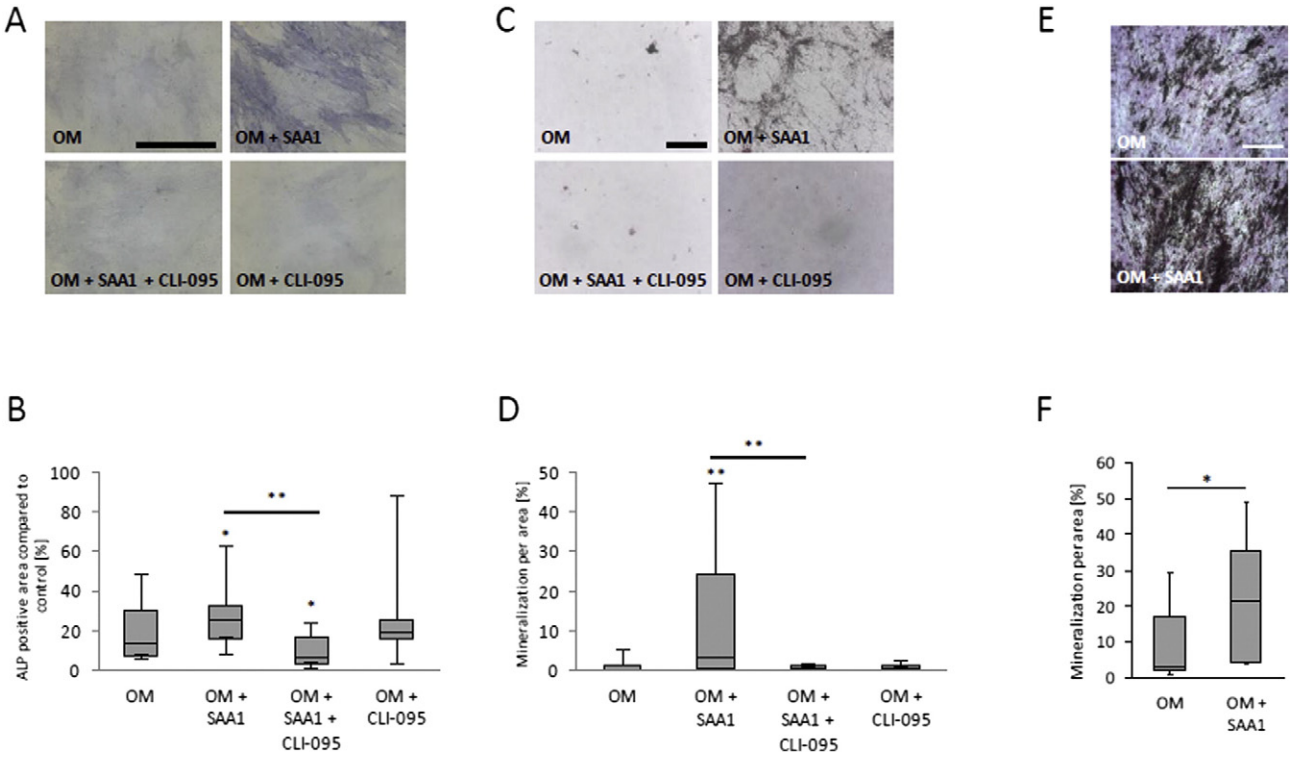

Fig. 5. Osteogenic differentiation and mineralization. Human MSCs were treated with osteogenic media (OM), OM + 1 μM rhSAA1, OM + 1 μM rhSAA1 + CLI-095 and OM + CLI-095 and ALP staining and the mineralized matrix per area was quantified. Panel (A) shows the representative images of ALP staining after 4 days. (B) Quantification of ALP staining (3 individual MSC donors, 6 images each) was performed after osteogenic differentiation for 4 days. Panel (C) shows the representative images of Alizarin Red S stained mineralized matrix after 7 days of osteogenic differentiation, length of bar represents 200 μM. Mineralization was quantified in 3 donors, 6 images each (D). (E) Human MSCs were treated with osteogenic media (OM) and OM + 1 μM rhSAA1 for 3 weeks and fixed and stained with Alizarin Red S. Representative images are shown, length of bar indicates 200 μm. (F) The amount of mineralized matrix per area was quantified for hMSC of 8 donors, 20 images each. (*$p < 0.05$ and **$p < 0.005$ by Wilcoxon signed-ranks test).

realized that WNT5A and ROR2, two key players in non-canonical wnt signaling and stimulation of osteogenic differentiation, are direct targets of TLR4 activation during osteogenic differentiation. Mechanistically this involved p38 and p65 phosphorylation and NFκB activation. We conclude from these data that in the early phase of bone regeneration and fracture healing the process of osteogenic differentiation is directly enhanced by TLR4 activation (confirmed by using the TLR4 inhibitor CLI-095) via autocrine and paracrine SAA1 and 2 production and their downstream targets, which again amplify the WNT5A induction demonstrated earlier (Rauner et al., 2012).

Mineralization is a hallmark of osteogenic differentiation, which is orchestrated by a set of genes and their substrates/products such as alkaline phosphatase and other ectophosphatases, phospho 1, ENPP and the calcification inhibitors osteopontin, FGF23 and osteocalcin, which propagate or inhibit crystallization to guarantee a coordinated mineralization process (Harmey et al., 2004). In order to characterize early and late osteogenic readouts under the influence of SAA we analyzed in vitro ALP activity and mineralization and we were able to demonstrate that early ALP activity and early and late mineralization are markedly enhanced in the presence of rhSAA1. This identical process could also be induced in osseous and extraosseous pathological calcification processes where SAA expression has been described. Hence SAA could also be tightly involved in calcifying inflammatory reactions such as we can see in atherosclerosis or in sclerosing bone metastases as frequently seen in prostate and breast cancer bone metastases, where also WNT5A is an important marker (Thiele et al., 2011; Zhang et al., 2012; Hansen et al., 2015; Le et al., 2005).

5. Conclusion

In summary we show here, that SAA is locally produced by mesenchymal stem cells both during in vitro aging and in early phases of in vitro osteogenic differentiation. SAA is capable of inducing the expression of an SASP resembling phenotype, which is either accelerating/enhancing osteogenic differentiation and mineralization, or induces the SASP that precedes replicative senescence. SAA may thus be involved in physiologic mineralization during bone formation, in pathologic calcification in degenerative age-associated diseases such as atherosclerosis and sclerosing bone metastases of e.g. breast and prostate cancers. SAA signals via p38 phosphorylation and p65/NFκB activation after binding to TLR4. Prolonged and pathological activation of this self-sustaining and self-amplifying loop can principally be targeted by TLR4 inhibitors but possibly with a substantial risk of interfering with principles of regeneration.

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